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(54) **COMPOSITIONS CRISTALLISABLES CONTENANT UN COMPLEXE A ASSOCIATION ENTRE DOMAINE DE PROTEASE NS3 DU VIRUS DE L'HEPATITE C ET NS4A ET CRISTAUX RESULTANTS**

(54) **CRYSTALLIZABLE COMPOSITIONS COMPRISING A HEPATITIS C VIRUS NS3 PROTEASE DOMAIN/NS4A COMPLEX AND CRYSTALS THEREBY OBTAINED**
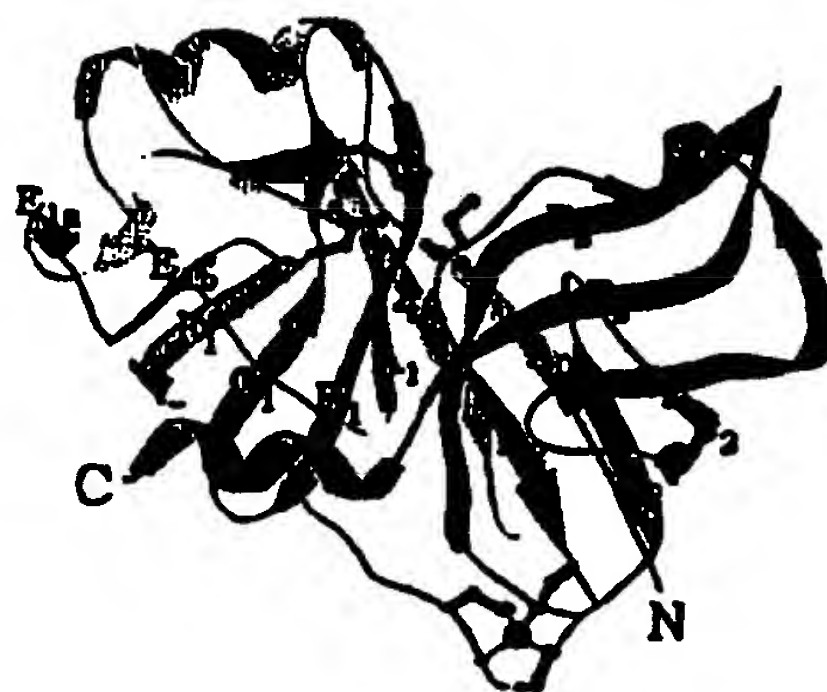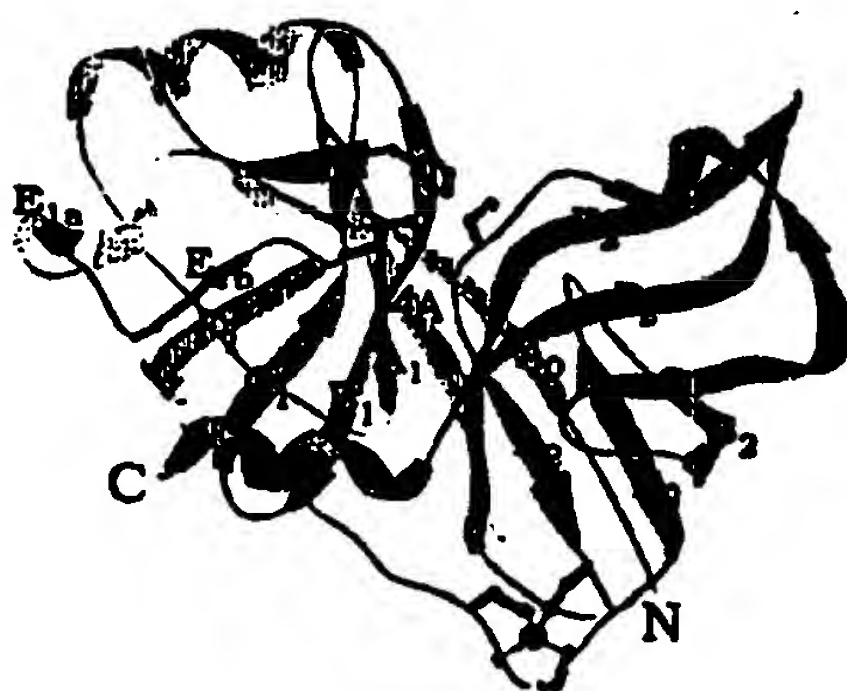
(57) Compositions et cristaux de protéase du virus de l'hépatite C associé en complexe avec son cofacteur viral. On décrit aussi des procédés relatifs à l'utilisation des coordonnées de structure de la protéase du virus de l'hépatite C, en complexe avec un NS4A synthétique, pour résoudre les problèmes liés à la structure de protéines ou de complexes de protéines similaires ou homologues.

(57) The present invention relates to compositions and crystals of a hepatitis C virus protease in complex with its viral cofactor. This invention also relates to methods of using the structure coordinates of hepatitis C virus protease in complex with a synthetic NS4A to solve the structure of similar or homologous proteins or protein complexes.

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(54) Title: CRYSTALLIZABLE COMPOSITIONS COMPRISING A HEPATITIS C VIRUS NS3 PROTEASE DOMAIN/NS4A COMPLEX AND CRYSTALS THEREBY OBTAINED

(57) Abstract

The present invention relates to compositions and crystals of a hepatitis C virus protease in complex with its viral cofactor. This invention also relates to methods of using the structure coordinates of hepatitis C virus protease in complex with a synthetic NS4A to solve the structure of similar or homologous proteins or protein complexes.

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CLAIMS

We claim:

1.   A crystallizable composition comprising a HCV NS3-like polypeptide complexed with a NS4A-like peptide.

2.   The composition according to claim 1, wherein the HCV NS3-like polypeptide is a NS3 protease domain polypeptide or a NS3 protease domain-like polypeptide.

3.   The composition according to claim 1, wherein the HCV NS3-like polypeptide is tNS3.

4.   The composition according to claim 1, 2 or 3, wherein the NS4A-like peptide is H-KKGSVVIVGRIVLSGKPAIIPKK-OH.

5.   A crystal comprising a HCV NS3-like polypeptide complexed with a NS4A-like peptide.

6.   The crystal according to claim 5, wherein the HCV NS3-like polypeptide is a NS3 protease domain polypeptide or a NS3 protease domain-like polypeptide.

7.   The crystal according to claim 5, wherein the HCV NS3-like polypeptide is tNS3.

8.   The crystal according to claim 5, 6, or 7, wherein the NS4A-like peptide is H-KKGSVVIVGRIVLSGKPAIIPKK-OH.

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9. The crystal according to claim 5, additionally comprising an inhibitor of HVC NS3.

10. A composition comprising a HCV NS3-like polypeptide complexed with a NS4A-like peptide, wherein the NS4A-like peptide is H-KKGSVVIVGRIVLSGKPAIIPKK-OH.

11. The composition according to claim 10, wherein the HCV NS3-like polypeptide is a NS3 protease domain polypeptide or a NS3 protease domain-like polypeptide.

12. The composition according to claim 10, wherein the HCV NS3-like polypeptide is tNS3.

13. A machine-readable data storage medium, comprising a data storage material encoded with machine readable data, wherein the data is defined by the structure coordinates of a tNS3/sNS4A complex according to Figure 3, or a homologue of said complex, wherein said homologue comprises backbone atoms that have a root mean square deviation from the backbone atoms of the complex of not more than 1.5A

14. The machine-readable data storage medium, according to claim 13, wherein said molecule or molecular complex is defined by the set of structure coordinates for tNS3/sNS4A according to Figure 3, or a homologue of said molecule or molecular complex, said homologue having a root mean square deviation from the backbone atoms of said amino acids of not more than 1.5 Å.

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15. A machine-readable data storage medium comprising a data storage material encoded with a first set of machine readable data comprising a Fourier transform of at least a portion of the structural coordinates for tNS3/sNS4A according to Figure 3; which, when combined with a second set of machine readable data comprising an X-ray diffraction pattern of a molecule or molecular complex of unknown structure, using a machine programmed with instructions for using said first set of data and said second set of data, can determine at least a portion of the structure coordinates corresponding to the second set of machine readable data, said first set of data and said second set of data.

16. A method of obtaining structural information about a molecule or a molecular complex of unknown structure by using the structure coordinates set forth in Figure 3, comprising the steps of:

a.   generating X-ray diffraction data from said crystallized molecule or molecular complex;

b.   applying at least a portion of the structure coordinates set forth in Figure 3 to said X-ray diffraction pattern to generate a three-dimensional electron density map of at least a portion of the molecule or molecular complex.

17. The method according to claim 16, wherein the molecule or molecular complex of unknown structure comprises a polypeptide selected from a NS3-like polypeptide in complex with a NS4A-like peptide.

CRYSTALLIZABLE COMPOSITIONS COMPRISING A HEPATITIS C VIRUS NS3 PROTEASE DOMAIN/NS4A COMPLEX AND CRYSTALS THEREBY OBTAINED

5   TECHNICAL FIELD OF INVENTION

The present invention relates to compositions and crystals of a hepatitis C virus protease in complex with its viral cofactor. This invention also relates
10   to methods of using the structure coordinates of hepatitis C virus protease in complex with a synthetic NS4A to solve the structure of similar or homologous proteins or protein complexes.

15   BACKGROUND OF THE INVENTION

Infection by hepatitis C virus (HCV) is a compelling human medical problem. HCV is recognized as the causative agent for most cases of non-A, non-B
20   hepatitis, with an estimated human seroprevalence of 1% globally [Choo, Q.-L. et al., "Isolation of a cDNA Clone Derived From a Blood-Borne Non-A, Non-B Viral Hepatitis Genome", Science, 244, pp. 359-362 (1989); Kuo, G. et al., "An Assay for Circulating Antibodies to
25   a Major Etiologic Virus of Human Non-A, Non-B Hepatitis", Science, 244, pp. 362-364 (1989); Purcell, R.H., "Hepatitis C virus: Historical perspective and current concepts", FEMS Microbiology Reviews, 14, pp. 181-192 (1994); Van der Poel, C.L., "Hepatitis C Virus.
30   Epidemiology, Transmission and Prevention in Hepatitis C virus. Current Studies in Hematology and Blood Transfusion, H.W. Reesink, Ed., (Basel: Karger), pp. 137-163 (1994)]. Four million individuals may be

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infected in the United States alone [Alter, M.J. and
Mast, E.E., "The Epidemiology of Viral Hepatitis in
the United States, Gastroenterol. Clin. North Am., 23,
pp. 437-455 (1994)].

5   Upon first exposure to HCV only about 20% of
infected individuals develop acute clinical hepatitis
while others appear to resolve the infection
spontaneously. In most instances, however, the virus
establishes a chronic infection that persists for

10   decades [Iwarson, S. "The Natural Course of Chronic
Hepatitis", FEMS Microbiology Reviews, 14, pp. 201-204
(1994)]. This usually results in recurrent and
progressively worsening liver inflammation, which often
leads to more severe disease states such as cirrhosis

15   and hepatocellular carcinoma [Kew, M.C., "Hepatitis C
and Hepatocellular Carcinoma", FEMS Microbiology
Reviews, 14, pp. 211-220 (1994); Saito, I., et al.
"Hepatitis C Virus Infection is Associated with the
Development cf Hepatocellular Carcinoma", Proc. Natl.

20   Acad. Sci. USA 87, pp. 6547-6549 (1990)]. Currently,
there are no broadly effective treatments for the
debilitating progression of chronic HCV.

              The HCV genome encodes a polyprotein of 3010-
3033 amino acids (Figure 1) [Choo, Q.-L., et al.

25   "Genetic Organization and Diversity of the Hepatitis C
Virus", Proc. Natl. Acad. Sci. USA, 88, pp. 2451-2455
(1991); Kato, N. et al., Molecular Cloning of the Human
Hepatitis C Virus Genome From Japanese Patients with
Non-A, Non-B Hepatitis", Proc. Natl. Acad. Sci. USA,

30   87, pp. 9524-9528 (1990); Takamizawa, A. et al.,
"Structure and Organization of the Hepatitis C Virus
Genome Isolated From Human Carriers", J. Virol., 65,
pp. 1105-1113 (1991)]. The HCV nonstructural (NS)

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proteins provide catalytic machinery for viral
replication. The NS proteins are derived by
proteolytic cleavage of the polyprotein
[Bartenschlager, R. et al., "Nonstructural Protein 3 of
5   the Hepatitis C Virus Encodes a Serine-Type Proteinase
Required for Cleavage at the NS3/4 and NS4/5
Junctions", J. Virol., 67, pp. 3835-3844 (1993);
Grakoui, A. et al. "Characterization of the Hepatitis C
Virus-Encoded Serine Proteinase: Determination of
10   Proteinase-Dependent Polyprotein Cleavage Sites", J.
Virol., 67, pp. 2832-2843 (1993); Grakoui, A. et al.,
Expression and Identification of Hepatitis C Virus
Polyprotein Cleavage Products", J. Virol., 67, pp.
1385-1395 (1993); Tomei, L. et al., "NS3 is a serine
15   protease required for processing of hepatitis C virus
polyprotein", J. Virol., 67, pp. 4017-4026 (1993)].

The HCV NS protein 3 (NS3) contains a serine
protease activity that helps process the majority of
the viral enzymes, and is thus considered essential for
20   viral replication and infectivity. It is known that
mutations in the yellow fever virus NS3 protease
decreases viral infectivity [Chambers, T.J. et. al.,
"Evidence that the N-terminal Domain of Nonstructural
Protein NS3 From Yellow Fever Virus is a Serine
25   Protease Responsible for Site-Specific Cleavages in the
Viral Polyprotein", Proc. Natl. Acad. Sci. USA, 87, pp.
8898-8902 (1990)]. The first 181 amino acids of NS3
(residues 1027-1207 of the viral polyprotein) have been
shown to contain the serine protease domain of NS3 that
30   processes all four downstream sites of the HCV
polyprotein (Figure 1) [C. Lin et al., "Hepatitis C
Virus NS3 Serine Proteinase: Trans-Cleavage

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Requirements and Processing Kinetics", J. Virol., 68,
pp. 8147-8157 (1994)].

NS3 is associated with a cofactor, NS4A.
NS4A seems critical to the activity of NS3, enhancing
5   the proteolytic efficiency of NS3 at all of the
cleavage sites. NS4A is a 54 residue amphipathic
peptide, with a hydrophobic N-terminus and a
hydrophilic C-terminus [Failla, C. et al., "Both NS3
and NS4A are Required for Proteolytic Processing of
10   Hepatitis C Virus Nonstructural Proteins", J. Virol.,
68, pp. 3753-3760 (1994)]. Its function appears
complex, possibly assisting in the membrane-
localization of NS3 and other viral replicase
components [Lin, C. et al. "A Central Region in the
15   Hepatitis C Virus NS4A Protein Allows Formation of an
Active NS3-NS4A Serine Proteinase Complex In Vivo and
In Vitro", J. Virol., 69, pp. 4373-4380 (1995b);
Shimizu, Y. et al., "Identification of the Sequence on
NS4A Required for Enhanced Cleavage of the NS5A/5B Site
20   by Hepatitis C Virus NS3 Protease", J. Virol., 70, pp.
127-132 (1996); Tanji, Y. et al., "Hepatitis C Virus-
Encoded Nonstructural Protein NS4A has Versatile
Functions in Viral Protein Processing", J. Virol., 69,
pp. 1575-1581 (1995)] but its best characterized
25   function is that of a cofactor for the NS3 protease.

The current understanding of HCV has not led
to satisfactory treatments for HCV infection. The
prospects for effective anti-HCV vaccines remain
uncertain. The only established therapy for HCV
30   disease is interferon treatment. However, interferons
have significant side effects [Janssen, H. L. A., et
al. "Suicide Associated with Alfa-Interferon Therapy
for Chronic Viral Hepatitis", J. Hepatol., 21, pp. 241-

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243 (1994)]; Renault, P.F. and Hoofnagle, J.H., "Side effects of alpha interferon. Seminars in Liver Disease 9, 273-277. (1989)] and induce long term remission in only a fraction (~ 25%) of cases [Weiland, O.

5   "Interferon Therapy in Chronic Hepatitis C Virus Infection", FEMS Microbiol. Rev., 14, pp. 279-288 (1994)]. Thus, there is a need for more effective anti-HCV therapies.

       The NS3 protease is considered a potential

10   target for antiviral agents. However, drug discovery efforts directed towards the NS3 protein have been hampered by the lack of structural information about NS3 and its complex with NS4A. Such structural information would provide valuable information in

15   discovery of HCV NS3 protease inhibitors. However, efforts to determine the structure of HCV NS3 protease have been hampered by difficulties in obtaining sufficient quantities of pure active enzyme [Steinkuhler, C. et al., "In Vitro Activity of

20   Hepatitis C Virus Protease NS3 Purified from Recombinant Baculovirus-Infected Sf9 Cells", J. Biol Chem., pp. 637-6273 (1996)]. There have been no crystals reported of any NS3 or NS3 protease domain protein. Thus, x-ray crystallographic analysis of such

25   proteins has not been possible.

                    SUMMARY OF THE INVENTION

              Applicants have solved this problem by

30   providing, for the first time, compositions comprising a hepatitis C virus (HCV) NS3 protease-like polypeptide complexed with a NS4A-like peptide and methods for making such compositions.

The invention also provides crystals of a HCV NS3 protease-like polypeptide/NS4A-like peptide complex and methods for making such crystals.

The invention also provides the structure
5   coordinates of a HCV NS3 protease-like polypeptide/NS4A-like peptide complex.

The invention also provides a method for determining at least a portion of the three-dimensional structure of molecules or molecular complexes which
10   contain at least some structurally similar features to a HCV NS3 serine protease domain.

BRIEF DESCRIPTION OF THE FIGURES

15   Figure 1 depicts HCV polyprotein processing. The locations of the HCV structural and nonstructural proteins are marked on a diagram of the 3011 amino acid polypeptide. Cleavages between the structural proteins by cellular signal peptidases are marked by asterisks.
20   Cleavage between NS2 and NS3 is mediated by the NS2/NS3 metallo-protease. The NS3 serine protease is responsible for cleavages between NS3 and NS4A, NS4A and NS4B, NS4B and NS5A, and NS5A and NS5B.

Figure 2 depicts stereo ribbon diagrams of
25   the NS3/NS4A complex. The view is into the active site cleft of the enzyme. Side-chains of active site residues His-1083, Asp-1107, and Ser-1165, along with Zn^{++} ligands Cys-1123, Cys-1125, and Cys-1171 are displayed in ball-and-stick representation. Zn^{++}, its
30   H_2O ligand, and the β-strand formed by NS4A are also shown.

Figure 3 lists the atomic structure coordinates for hepatitis C virus recombinant, truncated nonstructural protein 3 (hereafter referred to as tNS3) in complex with a synthetic peptide of the central region of the nonstructural protein 4A (hereafter referred to as sNS4A) as derived by X-ray diffraction from crystals of that complex (hereafter referred to as tNS3/sNS4A). The preparation of the complex is described in Examples 1 and 2. The following abbreviations are used in Figure 3:

"Atom type" refers to the element whose coordinates have been determined. Elements are defined by the first letter in the column except for zinc which is defined by the letters "Zn".

"X, Y, Z" crystallographically define the atomic position determined for each atom.

"B" is a thermal factor that measures movement of the atom around its atomic center.

"Occ" is an occupancy factor that refers to the fraction of the molecules in which each atom occupies the position specified by the coordinates. A value of "1" indicates that each atom has the same conformation, i.e., the same position, in all molecules of the crystal.

Figure 4 shows a diagram of a system used to carry out the instructions encoded by the storage medium of Figures 5 and 6.

Figure 5 shows a cross section of a magnetic storage medium.

Figure 6 shows a cross section of a optically-readable data storage medium.

DETAILED DESCRIPTION OF THE INVENTION

The following abbreviations are used throughout the application:

A = Ala = Alanine   T = Thr = Threonine

V = Val = Valine   C = Cys = Cysteine

L = Leu = Leucine   Y = Tyr = Tyrosine

I = Ile = Isoleucine   N = Asn = Asparagine

P = Pro = Proline   Q = Gln = Glutamine

F = Phe = Phenylalanine   D = Asp = Aspartic Acid

W = Trp = Tryptophan   E = Glu = Glutamic Acid

M = Met = Methionine   K = Lys = Lysine

G = Gly = Glycine   R = Arg = Arginine

S = Ser = Serine   H = His = Histidine

HCV = hepatitis C virus

5   Additional definitions are set forth in the specification where necessary.

In order that the invention described herein may be more fully understood, the following detailed description is set forth.

Applicants have solved the above problems by 10   providing, for the first time, crystallizable compositions comprising a HCV NS3 protease-like polypeptide in complex with a NS4A-like peptide.

Thus, in one embodiment of this invention is provided a composition comprising a hepatitis C virus 15   NS3-like polypeptide in complex with an NS4A-like peptide.

The HCV NS3-like polypeptide portion of the complex is any polypeptide which has the serine protease activity of the naturally occurring HCV NS3A 20   protease, particularly the ability to cleave the HCV

polyprotein. It includes HCV NS3, NS3 protease domain polypeptides and NS3 protease domain-like polypeptides.

As used herein, the terms "HCV NS3" and "NS3" refers to the hepatitis C virus nonstructural-3 protein as defined in Lin, C. et al., "Hepatitis C Virus NS3 Serine Proteinase: *Trans*-Cleavage Requirements and Processing Kinetics", J. Virol., 68, pp. 8147-8157 (1994).

The term "NS3 protease domain polypeptide" refers to a truncated, serine protease portion of NS3 as defined in [Bartenschlager, R. et al., "Nonstructural Protein 3 of the Hepatitis C Virus Encodes a Serine-Type Proteinase Required for Cleavage at the NS3/4 and NS4/5 Junctions", J. Virol., 67, pp. 3835-3844 (1993); Grakoui, A. et al. "Characterization of the Hepatitis C Virus-Encoded Serine Proteinase: Determination of Proteinase-Dependent Polyprotein Cleavage Sites", J. Virol., 67, pp. 2832-2843 (1993); Grakoui, A. et al., Expression and Identification of Hepatitis C Virus Polyprotein Cleavage Products", J. Virol., 67, pp. 1385-1395 (1993); Tomei, L. et al., "NS3 is a serine protease required for processing of hepatitis C virus polyprotein", J. Virol., 67, pp. 4017-4026 (1993)]. The disclosure of each of these documents is herein incorporated by reference.

The term "NS3 protease domain-like polypeptides" refers to polypeptides that differ from NS3 protease domain polypeptides by having amino acid deletions, substitutions, and additions, but which retain the serine protease activity of NS3.

Preferably, the NS3-like polypeptide in the compositions of this invention is tNS3, a recombinantly

produced hepatitis C virus protease domain protein that is prepared as described herein.

The NS4A-like peptide portion of the compositions of this invention is any peptide or peptide mimetic that is capable of acting as a NS4A cofactor for the NS3. These include NS4A, peptide fragments thereof and other peptides that differ from NS4A by having amino acid deletions, substitutions, and additions, while retaining the above-described activity.

As used herein the term "NS4A" refers to the hepatitis C virus nonstructural protein 4A which acts as a cofactor for NS3 protease [Failla, C. et al., "Both NS3 and NS4A are Required for Proteolytic Processing of Hepatitis C Virus Nonstructural Proteins". J. Virol. 68, pp. 3753-3760 (1994); Lin, C. et al., "Hepatitis C Virus NS3 Serine Proteinase: Trans-Cleavage Requirements and Processing Kinetics" J. Virol. 68, pp. 8147-8157 (1994b)]

Preferably, the NS4A-like peptide is sNS4A, the synthetic peptide H-KKGSVVIVGRIVLSGKPAIIPKK-OH. This peptide encompasses the essential NS3 protease domain residues of NS4A.

Both the NS3-like polypeptide and the NS4A-like peptide may be produced by any well-known method, including synthetic methods, such as solid phase, liquid phase and combination solid phase/liquid phase syntheses; recombinant DNA methods, including cDNA cloning, optionally combined with site directed mutagenesis; and/or purification of the natural products, optionally combined with enzymatic cleavage methods to produce fragments of naturally occurring NS3 and NS4A.

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According to a preferred embodiment, the compositions of this invention are crystallizable. In this preferred embodiment all of the preferred choices for the NS3-like polypeptide and the NS4A-like peptide are identical to those indicated above.

Advantageously, the crystallizable composition provided by this invention are amenable to x-ray crystallography. Thus, this invention also provides the three-dimensional structure of an HCV NS3-like polypeptide/NS4A-like peptide complex, specifically an HCV tNS3/sNS4A complex, at 2.5 Å resolution. Importantly, this has provided for the first time, information about the shape and structure of the NS3 protease domain.

The three-dimensional structure of the HCV tNS3/sNS4A complex of this invention is defined by a set of structure coordinates as set forth in Figure 3. The term "structure coordinates" refers to Cartesian coordinates derived from mathematical equations related to the patterns obtained on diffraction of a monochromatic beam of X-rays by the atoms (scattering centers) of an tNS3/sNS4A complex in crystal form. The diffraction data are used to calculate an electron density map of the repeating unit of the crystal. The electron density maps are then used to establish the positions of the individual atoms of the tNS3/sNS4A enzyme or enzyme complex.

Those of skill in the art will understand that a set of structure coordinates for an enzyme or an enzyme-complex or a portion thereof, is a relative set of points that define a shape in three dimensions. Thus, it is possible that an entirely different set of coordinates could define a similar or identical shape.

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Moreover, slight variations in the individual
coordinates will have little effect on overall shape.

The variations in coordinates discussed above
may be generated because of mathematical manipulations
5   of the structure coordinates. For example, the
structure coordinates set forth in Figure 3 could be
manipulated by crystallographic permutations of the
structure coordinates, fractionalization of the
structure coordinates, integer additions or
10   subtractions to sets of the structure coordinates,
inversion of the structure coordinates or any
combination of the above.

Alternatively, modifications in the crystal
structure due to mutations, additions, substitutions,
15   and/or deletions of amino acids, or other changes in
any of the components that make up the crystal could
also account for variations in structure coordinates.
If such variations are within an acceptable standard
error as compared to the original coordinates, the
20   resulting three-dimensional shape is considered to be
the same.

Various computational analyses are therefore
necessary to determine whether a molecule or molecular
complex or a portion thereof is sufficiently similar to
25   all or parts of the NS3-like polypeptide/NS4A-like
peptide structure described above as to be considered
the same. Such analyses may be carried out in current
software applications, such as the Molecular Similarity
application of QUANTA (Molecular Simulations Inc., San
30   Diego, CA) version 4.1, and as described in the
accompanying User's Guide.

The Molecular Similarity application permits
comparisons between different structures, different

conformations of the same structure, and different parts of the same structure. The procedure used in Molecular Similarity to compare structures is divided into four steps: 1) load the structures to be compared; 2) define the atom equivalences in these structures; 3) perform a fitting operation; and 4) analyze the results.

Each structure is identified by a name. One structure is identified as the target (i.e., the fixed structure); all remaining structures are working structures (i.e., moving structures). Since atom equivalency within QUANTA is defined by user input, for the purpose of this invention we will define equivalent atoms as protein backbone atoms (N, Cα, C and O) for all conserved residues between the two structures being compared. We will also consider only rigid fitting operations.

When a rigid fitting method is used, the working structure is translated and rotated to obtain an optimum fit with the target structure. The fitting operation uses an algorithm that computes the optimum translation and rotation to be applied to the moving structure, such that the root mean square difference of the fit over the specified pairs of equivalent atom is an absolute minimum. This number, given in angstroms, is reported by QUANTA.

For the purpose of this invention, any molecule or molecular complex that has a root mean square deviation of conserved residue backbone atoms (N, Cα, C, O) of less than 1.5 Å when superimposed on the relevant backbone atoms described by structure coordinates listed in Figure 3 are considered

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identical. More preferably, the root mean square
deviation is less than 1.0 Å.

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The term "root mean square deviation" means
the square root of the arithmetic mean of the squares
of the deviations from the mean. It is a way to
express the deviation or variation from a trend or
object. For purposes of this invention, the "root mean
square deviation" defines the variation in the backbone
of a protein or protein complex from the relevant

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portion of the backbone of the NS3-like polypeptide
portion of the complex as defined by the structure
coordinates described herein.

Once the structure coordinates of a protein
crystal have been determined they are useful in solving

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the structures of other crystals.

Thus, in accordance with the present
invention, the structure coordinates of a NS3-like
polypeptide/NS4A-like peptide complex, and in
particular a tNS3/sNS4A complex, and portions thereof

20

is stored in a machine-readable storage medium. Such
data may be used for a variety of purposes, such as
drug discovery and x-ray crystallographic analysis or
protein crystal.

Accordingly, in one embodiment of this

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invention is provided a machine-readable data storage
medium comprising a data storage material encoded with
the structure coordinates set forth in Figure 3.

Figure 4 demonstrates one version of these
embodiments. System 10 includes a computer 11

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comprising a central processing unit ("CPU") 20, a
working memory 22 which may be, e.g, RAM (random-access
memory) or "core" memory, mass storage memory 24 (such
as one or more disk drives or CD-ROM drives), one or

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more cathode-ray tube ("CRT") display terminals 26, one or more keyboards 28, one or more input lines 30, and one or more output lines 40, all of which are interconnected by a conventional bidirectional system
5   bus 50.

Input hardware 36, coupled to computer 11 by input lines 30, may be implemented in a variety of ways. Machine-readable data of this invention may be inputted via the use of a modem or modems 32 connected
10   by a telephone line or dedicated data line 34. Alternatively or additionally, the input hardware 36 may comprise CD-ROM drives or disk drives 24. In conjunction with display terminal 26, keyboard 28 may also be used as an input device.

15   Output hardware 46, coupled to computer 11 by output lines 40, may similarly be implemented by conventional devices. By way of example, output hardware 46 may include CRT display terminal 26 for displaying a graphical representation of a binding
20   pocket of this invention using a program such as QUANTA as described herein. Output hardware might also include a printer 42, so that hard copy output may be produced, or a disk drive 24, to store system output for later use.

25   In operation, CPU 20 coordinates the use of the various input and output devices 36, 46, coordinates data accesses from mass storage 24 and accesses to and from working memory 22, and determines the sequence of data processing steps. A number of
30   programs may be used to process the machine-readable data of this invention. Such programs are discussed in reference to the computational methods of drug discovery as described herein. Specific references to

components of the hardware system 10 are included as appropriate throughout the following description of the data storage medium.

Figure 5 shows a cross section of a magnetic
5   data storage medium 100 which can be encoded with a
machine-readable data that can be carried out by a
system such as system 10 of Figure 4. Medium 100 can
be a conventional floppy diskette or hard disk, having
a suitable substrate 101, which may be conventional,
10 and a suitable coating 102, which may be conventional,
on one or both sides, containing magnetic domains (not
visible) whose polarity or orientation can be altered
magnetically. Medium 100 may also have an opening (not
shown) for receiving the spindle of a disk drive or
15 other data storage device 24.

The magnetic domains of coating 102 of medium
100 are polarized or oriented so as to encode in manner
which may be conventional, machine readable data such
as that described herein, for execution by a system
20 such as system 10 of Figure 4.

Figure 6 shows a cross section of an
optically-readable data storage medium 110 which also
can be encoded with such a machine-readable data, or
set of instructions, which can be carried out by a
25 system such as system 10 of Figure 4. Medium 110 can
be a conventional compact disk read only memory
(CD-ROM) or a rewritable medium such as a
magneto-optical disk which is optically readable and
magneto-optically writable. Medium 100 preferably has
30 a suitable substrate 111, which may be conventional,
and a suitable coating 112, which may be conventional,
usually of one side of substrate 111.

In the case of CD-ROM, as is well known, coating 112 is reflective and is impressed with a plurality of pits 113 to encode the machine-readable data. The arrangement of pits is read by reflecting laser light off the surface of coating 112. A protective coating 114, which preferably is substantially transparent, is provided on top of coating 112.

In the case of a magneto-optical disk, as is well known, coating 112 has no pits 113, but has a plurality of magnetic domains whose polarity or orientation can be changed magnetically when heated above a certain temperature, as by a laser (not shown). The orientation of the domains can be read by measuring the polarization of laser light reflected from coating 112. The arrangement of the domains encodes the data as described above.

For the first time, the present invention permits the use of structure-based or rational drug design techniques to design, select, and synthesize chemical entities, including inhibitory compounds that are capable of binding to HCV NS3, NS4A, NS3/NS4A complex, or any portion thereof.

One particularly useful drug design technique enabled by this invention is iterative drug design. Iterative drug design is a method for optimizing associations between a protein and a compound by determining and evaluating the three-dimensional structures of successive sets of protein/compound complexes.

Those of skill in the art will realize that association of natural ligands or substrates with the binding pockets of their corresponding receptors or

enzymes is the basis of many biological mechanisms of
action. The term "binding pocket", as used herein,
refers to a region of a molecule or molecular complex,
that, as a result of its shape, favorably associates

5   with another chemical entity or compound. Similarly,
many drugs exert their biological effects through
association with the binding pockets of receptors and
enzymes. Such associations may occur with all or any
parts of the binding pockets. An understanding of such

10   associations will help lead to the design of drugs
having more favorable associations with their target
receptor or enzyme, and thus, improved biological
effects. Therefore, this information is valuable in
designing potential ligands or inhibitors of receptors

15   or enzymes, such as inhibitors of HCV NS3-like
polypeptides, and more importantly HCV NS3.

          The term "associating with" refers to a
condition of proximity between chemical entities or
compounds, or portions thereof. The association may be

20   non-covalent -- wherein the juxtaposition is
energetically favored by hydrogen bonding or van der
Waals or electrostatic interactions -- or it may be
covalent.

          In iterative drug design, crystals of a

25   series of protein/compound complexes are obtained and
then the three-dimensional structures of each complex
is solved. Such an approach provides insight into the
association between the proteins and compounds of each
complex. This is accomplished by selecting compounds

30   with inhibitory activity, obtaining crystals of this
new protein/compound complex, solving the three-
dimensional structure of the complex, and comparing the
associations between the new protein/compound complex

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and previously solved protein/compound complexes. By observing how changes in the compound affected the protein/compound associations, these associations may be optimized.

5   In some cases, iterative drug design is carried out by forming successive protein-compound complexes and then crystallizing each new complex. Alternatively, a pre-formed protein crystal is soaked in the presence of an inhibitor, thereby forming a
10   protein/compound complex and obviating the need to crystallize each individual protein/compound complex. Advantageously, the HCV NS3-like polypeptide/NS4A-like peptide crystals, and in particular the tNS3/sNS4A crystals, provided by this invention may be soaked in
15   the presence of a compound or compounds, such as NS3 protease inhibitors, to provide NS3-like polypeptide/NS4A-like peptide /compound crystal complexes.

          As used herein, the term "soaked" refers to a
20   process in which the crystal is transferred to a solution containing the compound of interest.

          In another embodiment of this invention is provided a method for preparing a composition comprising a NS3-like polypeptide protein comprising
25   the steps described in Examples 1 and 2. Preferably, the composition comprises a NS3-like polypeptide in complex with a NS4A-like peptide.

          The structure coordinates set forth in Figure 3 can also be used to aid in obtaining structural
30   information about another crystallized molecule or molecular complex. This may be achieved by any of a number of well-known techniques, including molecular replacement.

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The structure coordinates set forth in Figure
3 can also be used for determining at least a portion
of the three-dimensional structure of molecules or
molecular complexes which contain at least some

5   structurally similar features to HCV NS3.   In
particular, structural information about another
crystallized molecule or molecular complex may be
obtained.   This may be achieved by any of a number of
well-known techniques, including molecular replacement.

10   Therefore, in another embodiment this
invention provides a method of utilizing molecular
replacement to obtain structural information about a
crystallized molecule or molecular complex whose
structure is unknown comprising the steps of:

15   a) generating an X-ray diffraction pattern
from said crystallized molecule or molecular complex;
and

b) applying at least a portion of the
structure coordinates set forth in Figure 3 to the

20   X-ray diffraction pattern to generate a
three-dimensional electron density map of the molecule
or molecular complex whose structure is unknown.

Preferably, the crystallized molecule or
molecular complex comprises a NS3-like polypeptide and

25   a NS4A-like peptide.   More preferably, the crystallized
molecule or molecular complex is obtained by soaking a
crystal of this invention in a solution.

By using molecular replacement, all or part
of the structure coordinates of the tNS3/sNS4A complex

30   provided by this invention (and set forth in Figure 3)
can be used to determine the structure of a
crystallized molecule or molecular complex whose
structure is unknown more quickly and efficiently than
attempting to determine such information _ab_ _initio_.

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Molecular replacement provides an accurate estimation of the phases for an unknown structure. Phases are a factor in equations used to solve crystal structures that can not be determined directly.

5   Obtaining accurate values for the phases, by methods other than molecular replacement, is a time-consuming process that involves iterative cycles of approximations and refinements and greatly hinders the solution of crystal structures. However, when the

10   crystal structure of a protein containing at least a homologous portion has been solved, the phases from the known structure provide a satisfactory estimate of the phases for the unknown structure.

Thus, this method involves generating a

15   preliminary model of a molecule or molecular complex whose structure coordinates are unknown, by orienting and positioning the relevant portion of the tNS3/sNS4A complex according to Figure 3 within the unit cell of the crystal of the unknown molecule or molecular

20   complex so as best to account for the observed X-ray diffraction pattern of the crystal of the molecule or molecular complex whose structure is unknown. Phases can then be calculated from this model and combined with the observed X-ray diffraction pattern amplitudes

25   to generate an electron density map of the structure whose coordinates are unknown. This, in turn, can be subjected to any well-known model building and structure refinement techniques to provide a final, accurate structure of the unknown crystallized molecule

30   or molecular complex [E. Lattman, "Use of the Rotation and Translation Functions", in Meth. Enzymol., 115, pp. 55-77 (1985); M. G. Rossmann, ed., "The Molecular Replacement Method", Int. Sci. Rev. Ser., No. 13, Gordon & Breach, New York (1972)].

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The structure of any portion of any crystallized molecule or molecular complex that is sufficiently homologous to any portion of the tNS3/sNS4A complex can be solved by this method.

5   In a preferred embodiment, the method of molecular replacement is utilized to obtain structural information about a molecule or molecular complex, wherein the complex comprises a NS3-like polypeptide. Preferably the NS3-like polypeptide is tNS3 or
10   homologue thereof.

The structure coordinates of tNS3/sNS4A as provided by this invention are particularly useful in solving the structure of other crystal forms of NS3-like polypeptide, preferably other crystal forms of
15   tNS3; NS3-like polypeptide/NS4A-like peptide, preferably tNS3/sNS4A; or complexes comprising any of the above.

The structure coordinates are also particularly useful to solve the structure of crystals
20   of NS3-like polypeptide/NS4A-like peptide complexes, particularly tNS3/sNS4A, co-complexed with a variety of chemical entities. This approach enables the determination of the optimal sites for interaction between chemical entities, including interaction of
25   candidate NS3 inhibitors with NS3 or the NS3/NS4A complex. For example, high resolution X-ray diffraction data collected from crystals exposed to different types of solvent allows the determination of where each type of solvent molecule resides. Small
30   molecules that bind tightly to those sites can then be designed and synthesized and tested for their NS3 inhibition activity.

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All of the complexes referred to above may be studied using well-known X-ray diffraction techniques and may be refined versus 1.5-3 Å resolution X-ray data to an R value of about 0.20 or less using computer
5   software, such as X-PLOR [Yale University, ©1992, distributed by Molecular Simulations, Inc.; see, e.g., Blundell & Johnson, supra; Meth. Enzymol., vol. 114 & 115, H. W. Wyckoff et al., eds., Academic Press (1985)]. This information may thus be used to optimize
10   known NS3 inhibitors, and more importantly, to design new NS3 inhibitors.

In order that this invention be more fully understood, the following examples are set forth. These examples are for the illustrative purposes only
15   and are not to be construed as limiting the scope of this invention in any way.

EXAMPLE 1
Expression and Purification of tNS3
20   The truncated NS3 serine protease domain (tNS3) was cloned from a cDNA of the hepatitis C virus H strain [Grakoui, A. et al., "Expression and Identification of Hepatitis C Virus Polyprotein Cleavage Products", J. Virol., 67, pp. 1385-1395
25   (1993)]. The first 181 amino acids of NS3 (residues 1027-1207 of the viral polyprotein) have been shown to contain the serine protease domain of NS3 that processes all four downstream sites of the HCV polyprotein [Lin, C., et al., Hepatitis C Virus NS3
30   Serine Proteinase: Trans-Cleavage Requirements and Processing Kinetics", J. Virol. 68, pp. 8147-8157 (1994b)], so we expressed a $(His)_6$-fusion protein based on this tNS3. The plasmid pET-BS(+)/HCV/T7-NS3$_{181}$-His

was derived from pTM3/HCV/1027-1207 (NS3$_{181}$) (Id.), by
using polymerase chain reaction to introduce epitope
tags and new restriction sites. A T7-tag (ASMTGGQQMG),
from the N-terminus of the gene 10 protein of the T7

5   bacteriophage
[Tsai, D.E. et al., "*In Vitro* Selection of an RNA
Epitope Immunologically Cross-Reactive With a Peptide",
Proc. Natl. Acad. Sci. USA, 89, pp. 8864-8868 (1992)],
was placed at the N-terminus of the tNS3 domain. Two

10   linker residues (GS) were placed at the tNS3 C-
terminus, followed by the (His)$_6$-tag. *E.coli*
JM109(DE3) cells, freshly transformed with the pET-
BS(+)/HCV/T7-NS3$_{181}$-His plasmid, were grown at 37 °C in
complex media supplement with 100 µg/ml ampicillin, in

15   a 10 L fermentor (Braun). When the cell density
reached an OD$_{600}$ of 3-4 the temperature of the culture
was rapidly reduced to 30 °C, and induction was
immediately initiated by the addition of 1 mM IPTG.
Cells were harvested at 2 h post-induction, and flash

20   frozen at -70 °C prior to purification.

      The tNS3 was purified from the soluble
fraction of the recombinant *E.coli* lysates as follows,
with all procedures being performed at 4 °C unless
stated otherwise. Cell paste (75-100g) was resuspended

25   in 15 volumes of 50 mM HEPES, 0.3 M NaCl, 10% glycerol,
0.1% ß-octyl glucoside, 2 mM ß-mercaptoethanol, pH 8.0.
Cells were ruptured using a microfluidizer and the
homogenate was clarified by centrifugation at 100,000 x
g for 30 min. The supernatant was brought to 50 mM

30   HEPES, 20 mM imidazole, 0.3 M NaCl, 27.5% glycerol,
0.1% ß-octyl- glucoside, 2 mM ß-mercaptoethanol, pH
8.0, and applied at 1.0 ml/min to a 7.0 ml Ni-Agarose

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affinity column, equilibrated in the same buffer.
After loading, the column was washed with 10-15 volumes
of equilibration buffer and the bound proteins were
eluted with equilibration buffer containing 0.35 M

5   imidazole. The protein was then size-fractionated on
two columns in series (each 2.6 cm x 90 cm) packed with
Pharmacia high resolution S100 resin and equilibrated
with 25 mM HEPES, 0.3M NaCl, 10% glycerol, 0.1% ß-
octylglucoside, 2 mM ß-mercaptoethanol, pH 8.0. The

10 tNS3 fractions, identified by SDS-PAGE, were pooled and
concentrated to 1 mg/ml using a Amicon Centriprep-10,
and stored at -70°C. The tNS3 was thawed slowly on ice
and the NS4A peptide (dissolved in the size-exclusion
chromatography buffer) was added at a tNS3:NS4A-peptide

15 molar ratio of 1:2. The sample was then diluted 2.5-
fold with 15 mM MES, 0.5 M NaCl, 20 mM ß-
mercaptoethanol, pH6.5, and concentrated to ~2 ml (~ 2
mg/ml) by ultrafiltration. The sample was then diluted
2-fold with the pH 6.5 buffer and concentrated again to

20 ~2 ml. This dilution process was repeated until it
gave a >40-fold dilution of the original buffer
constituents. The protein sample was then concentrated
to 13.0 mg/ml and centrifuged at ~300,000 x g for 20
min at 4 °C. Concentrations of the pure tNS3 and

25 tNS3/4A complex were determined by UV absorption
spectroscopy, using a molar absorption coefficient
(A_{280}) of 17,700 $M-1.cm-1$.

EXAMPLE 2
4A Peptide Synthesis and Purification

30   The HCV NS4A peptide was synthesized to span
residues Gly21 to Pro39 of the viral cofactor (residues

1678 to 1696 of the HCV polyprotein), which
incorporates the essential region reported to be
essential for NS3 stimulation [Lin, C. et al. "A
Central Region in the Hepatitis C Virus NS4A Protein
5   Allows Formation of an Active NS3-NS4A Serine
Proteinase Complex _In Vivo_ and _In Vitro_", J. Virol. 69,
pp. 4373-4380 (1995)]. Lysine residues were added to
the termini to assist aqueous solubility, and a serine
residue was substituted for Cys22 (residue 1679 of the
10   polyprotein of the HCV H strain). The peptide (H-
KKGSVVIVGRIVLSGKPAIIPKK-OH · TFA salt) was prepared by
the solid-phase peptide synthesis (Applied Biosystems
433A) beginning with N^a-Fmoc, N^e-Boc-Lys Wang resin.
N^a-Fmoc-protected amino acids were added sequentially
15   using HBTU (2-(1H-benzotriazol-1-yl)1,1,3,3-
tetramethyluronium hexafluorophosphate) with HOBt (1-
hydroxybenzotriazole hydrate) as coupling agents in N-
methylpyrrolidinone. Cleavage from the resin and
global deprotection were accomplished with 95%
20   trifluoroacetic acid and 5% water at room temperature
for 1.5 hr (15 ml/ g resin). The peptide was purified
by preparative HPLC on a Waters Delta Pak C18, 15 μm,
300Å column (30 mm x 300 mm) eluting with a linear
gradient of acetonitrile (15-40%) in 0.1% aqueous
25   trifluoroacetic acid over 35 min (flow rate of 22
ml/min). Peptide purity was confirmed by analytical
HPLC. The sequence was confirmed by direct N-terminal
sequence analysis and matrix-assisted laser desorption
mass spectrometry (Kratos MALDI I), which showed the
30   correct $(M + H)^+$ and $(M + Na)^+$ molecular ions.

EXAMPLE 3
Crystallization and Data Collection

Crystals of the tNS3/NS4A complex were grown
by hanging-drop vapor diffusion over a reservoir of 0.1
M MES, 1.8 M NaCl, 0.1 M sodium/potassium phosphate, 10
mM ß-mercaptoethanol, pH 6.5. The crystals grew over
the course of 2-3 weeks, to final dimensions of about
0.1 x 0.1 x 0.25 mm. The rhombohedral crystals used in
this study belonged to space group R32, with unit cell
dimensions a=b=225.0Å, and c=75.5Å, and contained two
tNS3/NS4A complexes per asymmetric unit.

Statistics for data collection, heavy atom
refinement, and crystallographic refinement are given
in Table 1. All heavy atom soaks were done in hanging-
drops over the same reservoir as used for
crystallization. Crystals were transferred to a
stabilizing solution (50 mM MES, 2.0 M NaCl, 0.1 M
sodium/potassium phosphate, 10 mM ß-mercaptoethanol,
and 20% glycerol, pH 6.2) and then frozen in a dry
nitrogen gas stream at 100 K (Molecular Structure
Corp., Houston, TX) for data collection. Data was
acquired by oscillation photography on a Rigaku R-AXIS
IIC phosphor imaging area detector mounted on a Rigaku
RU200 rotating anode generator (MSC), operating at 50kV
and 100mA. Measured intensities were integrated,
scaled, and merged using the HKL software package (Z.
Otwinowski and W. Minor).

EXAMPLE 4
Phasing, Model Building and Refinement

Heavy atom positions were located by
inspection and confirmed with difference Fourier
syntheses. Heavy atom parameters were refined and

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phases computed to 3.1Å using the program PHASES
[Furey, W. and Swaminathan, S. "PHASES-95: a program
package for the processing and analysis of diffraction
data from macromolecules", Meth. Enzymol., (1996). MIR
5   phases were improved and extended to 2.7Å by cycles of
solvent flattening [Wang, B.C., "Resolution of Phase
Ambiguity in Macromolecular Crystallography", Methods
in Enzymol. 115, pp. 90-112 (1985)] combined with
histogram matching [Zhang, K.Y.J. and Main, P., "The
10   Use of Sayre's Equation With Solvent Flattening and
Histogram Matching for Phase Extension and Refinement
of Protein Structures", Acta Crystallogr., A46, pp.
377-381 (1990)] using the CCP4 crystallographic package
(Collaborative Computation Project, 1994). The
15   resulting electron density map displayed nearly
continuous density for the protein backbone as well as
strong side chain density. Approximately 80% of the
model could be unambiguously built into this map
(QUANTA 4.1, Molecular Simulations), and a single round
20   of simulated annealing refinement in X-PLOR [Brunger,
A. T., "X-PLOR: A System for X-Ray Crystallography and
NMR", New Haven, Connecticut: Department of Molecular
Biophysics and Biochemistry, Yale University (1993)]
brought the R-factor to 29% and free R value to 33%
25   [Brunger, A. T., "Free R Value: A Novel Statistical
Quantity for Assessing the Accuracy of Crystal
Structures", Nature, 355, pp. 472-475 (1992)]. The
remainder of the model was built and refined in several
steps, by first extending the resolution to 2.5Å and
30   then adding well-ordered water molecules. A final
round of positional and individual temperature factor
refinement brought the R-factor to 21.6% (free R value
26.1%) for 26,652 reflections between 6.0 and 2.5Å

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(F>1sF). The current model consisted of tNS3 residues 1055-1206 and NS4A residues 1678-1693 in complex A, and tNS3 residues 1028-1206 and NS4A residues 1678-1696 for complex B (polyprotein numbering, with 2 zinc atoms

5   and 130 water molecules. A Ramachandran plot for the final model contained 91% of the residues in the most favored regions and 0% in disallowed or generously-allowed regions. The rms deviations from ideality were 0.007Å for bond lengths and 1.47° for bond angles.

10   While we have described a number of embodiments of this invention, it is apparent that our basic examples may be altered to provide other embodiments which utilize the products and processes of this invention. Therefore, it will be appreciated that

15   the scope of this invention is to be defined by the appended claims rather than by the specific embodiments which have been represented by way of example.